



VANM213.001AUS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Remacle et al.
Appl. No. : 09/817,014
Filed : March 23, 2001
For : IDENTIFICATION OF BIOLOGICAL
(MICRO)ORGANISMS BY
DETECTION OF THEIR
HOMOLOGOUS NUCLEOTIDE
SEQUENCES ON ARRAYS
Examiner : Calamita, Heather
Group Art Unit : 1637

DECLARATION UNDER 37 C.F.R. §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate that the claimed invention unexpectedly provides the ability to rapidly determine which of several nucleic acids having greater than 30% homology with one another is present in a sample with high sensitivity and specificity.
2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.
3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.
4. The claimed invention provides accurate discrimination between homologous sequences and yields results within 60 and even within 30 minutes of the conclusion of the hybridization step. In order to provide specificity, the claimed invention employs a capture molecule comprising a short sequence which is between about 15 and about 40 bases in length and which is specific for the target sequence. In order to provide specific features related to the invention as

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explained below, the sequence specific for the target sequence is linked to a nucleic acid spacer which is at least 40 nucleotides in length.

5. The present invention was designed to overcome a problem in prior art methods for identifying and/or quantifying the biological organisms. The problem with identifying homologous sequences on an array is that one has to have good sensitivity to be able to detect the signal combined with good specificity to be able to distinguish among different but homologous sequences present in a sample. The process of identifying should not be overly time consuming. The problem in the prior art was that in order to obtain good specificity of detection of long target molecules, short capture probes attached to a solid support without a spacer could be employed. However, such a method would result in the detected signal being too low, i.e. the sensitivity of the method will be too low. Moreover, the kinetics of the reaction are poor, because the two strands of the long target sequence will preferentially re-anneal to one another rather than hybridizing to short capture nucleotide sequences immobilized on a surface, resulting in low sensitivity. On the other hand, if the capture probe is long, then the method of detection of long target probes is sensitive, but has low specificity due to non-specific cross-reaction of the capture probe with the other species of the target molecules present in a sample.

6. To avoid the kinetic problem, prior art assays have typically used short capture sequences for short targets or long capture sequences for long targets. However, either option necessarily invokes one of the disadvantages discussed above.

7. The inventors unexpectedly found that if a single-stranded capture molecule has a nucleic acid spacer of at least 40 bases long connected to the surface of the solid support, then the detection of long target sequences (100-800 bp) using short specific capture probes gains in sensitivity without losing high specificity, AND the detection of long target sequences using overall long capture probes gains in specificity without losing high sensitivity. Keeping the detection sensitivity together with the specificity for detection of homologous amplicons was an unexpected result of the present invention.

8. The ability of the claimed invention to accurately discriminate between homologous sequences and to rapidly yield results is extremely valuable in commercial applications. The product to be used with the methods of the current invention is expected to be put on the market

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soon to achieve fast and safe analysis of genetically modified organisms in foodstuff. The product to be commercialized is referred to as the Eppendorf DualChip® GMO microarray (see Exhibit 1).

9. In order to provide high sensitivity, the claimed invention utilizes a relatively long double-stranded target sequence which is between 100 and 800 bases in length. The use of such long sequences allows to increase the sensitivity of the methods since many labels can be incorporated into a such a long DNA fragment during the amplification step. Also, there is no need to cut the amplicons into fragments for the selection which makes the method of the invention easy to use since the PCR solution can be directly hybridized on the array without any further treatment. However, such long target sequences do not hybridize as efficiently to short capture sequences as to long capture nucleotide sequences. The present invention overcomes this problem by linking the short capture nucleotide sequence to a nucleic acid spacer which is at least 40 nucleotides in length.

10. Using a longer capture nucleotide sequence would enable the capture nucleotide sequence to more efficiently hybridize to long target sequences. However, use of long capture nucleotide sequences results in reduced specificity. The present invention overcomes this problem by linking a short capture nucleotide sequence which is about 15 to about 40 nucleotides in length to a nucleic acid spacer which is at least 40 nucleotides in length.

11. Exhibits 2-6 show the data obtained by the inventors in support of the advantages of the claimed method.

12. Exhibit 2 shows that hybridization of long double-stranded target molecules to short capture probes which are not linked to a spacer is specific, but the obtained signal is too low. In the experiment of Exhibit 2, double-stranded target molecules of 489 bp of the *S. epidermidis* *FemA* gene were hybridized to short capture probes from the *FemA* genes from *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. saprophyticus* which were 27 bases in length and which lacked a nucleotide spacer. The protocol provided in Example 2 of the present application was used to conduct the analysis. As depicted in Exhibit 2, a signal is only observed on the *S. epidermidis* capture probe but the signal value is very low (signal value = 13) and almost

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undetectable. Thus, use of a short capture probe which lacks a spacer provides specificity but not sensitivity.

13. Exhibit 3 shows that hybridization of long double-stranded target molecules to long capture probes without a spacer gives a good signal but is non-specific. Double-stranded target molecules of 101 bp of the *S. epidermidis* *FemA* gene were hybridized to long capture probes of 101 bases for *S. epidermidis*, 108 bases for *S. saprophyticus*, 139 bases for *S. aureus*, 128 bases for *S. haemolyticus* and 118 bases for *S. hominis*. The protocol of Example 1 of the present application was followed in this experiment. As depicted in Exhibit 3, the target *S. epidermidis* amplicons were able to specifically bind to the capture probe for *S. epidermidis* with a high signal value (152), but they also non-specifically bound to the other 4 capture probes (signal value = 144, 9, 13 and 20 respectively for the *S. saprophyticus*, *S. aureus*, *S. haemolyticus* and *S. hominis* capture nucleotide sequences). In conclusion, the use of long capture sequences with long target sequences provides sensitivity but not specificity.

14 Exhibit 4 shows that when a short capture probe includes a nucleic acid spacer, it hybridizes with its target with high specificity and also gives a good signal. In fact, the signal level is similar to that obtained with a long capture probe that does not include a nucleic acid spacer. In the experiment of Exhibit 4, a double stranded target molecule of 587 bp from the *S. aureus* *FemA* gene was hybridized to long capture probes comprising of a spacer of 40 nucleotides and a short sequence of 27 nucleotides specific for the *FemA* genes of different *Staphylococci* species. The protocol of Examples 4 and 7 of the present application were utilized for this experiment and the resulting signals were quantitated. A consensus capture probe of 489 nucleotides which hybridizes to the *FemA* gene of all of the foregoing species of *Staphylococcus* was included as a positive control. As depicted in Exhibit 4, a signal was only observed on the capture probe specific for *S. aureus* and on the consensus capture probe.

15. In theory, the rate of hybridization is proportional to the square root of the smaller sequence taking part to the hybridization (i.e. the sequence of the capture probe of 489 or 27 bases). Accordingly, the reassociation of long amplicons of 587 bases or the hybridization of the amplicon on the capture probe of 489 bases should be much faster than the hybridization of the amplicon on a capture probe of 27 bases. However, unexpectedly, the signal value obtained with the *S. aureus* specific capture probe comprising 27 nucleotides complementary to the target

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was the same as that obtained with the 489 nucleotide consensus probe the signal values were very high (signal values = 152 and 158 respectively for *S. aureus* and the consensus capture nucleotide sequences). The signal values obtained with the 27 nucleotide capture probe linked to the spacer demonstrates the high sensitivity of the claimed invention.

16. Exhibit 5 shows that even when a plurality of amplified targets is present in a sample, the use of a short specific capture probe linked to a spacer of at least 40 nucleotides, allows obtaining specific signals of high values (i.e. the method is sensitive and specific) Double stranded targets of 587 bp of the *S. gallinarum FemA* gene and the *S. saprophyticus FemA* gene were generated by amplifying the two targets using consensus primers as described in the specification of the present application. The amplified targets were hybridized to capture probes comprising a spacer of 40 nucleotides and a short (27 bases in length) sequence specific for the target sequences using the protocols of Examples 4 and 7 of the present application and the resulting signals were quantitated. A signal was only observed on the specific capture probes of *S. gallinarum* and *S. saprophyticus* and the signal values were very high (signal value = 169, 136 and 167 respectively for *S. gallinarum*, *S. saprophyticus* and the consensus capture nucleotide sequences). There is an unambiguous detection of the two bacterial species present in the sample. In conclusion, the assay is both sensitive and specific even when a plurality of targets is present in the sample.

17. Exhibit 6 shows that the length of the spacer is important for the sensitivity of detection of the hybridization of a long double-stranded target on short specific capture molecules linked to a nucleic acid spacer. A long double stranded target of 587 bp of the *S. epidermidis FemA* gene was hybridized to capture probes having spacers of different lengths (20, 40, 85 and 95 bases) linked to a sequence specific for the target which was 27 bases in length. As shown in Exhibit 6, the rate of hybridization was increased by a factor of 5 for a spacer portion of 95 nucleotides, as compared to a spacer portion of 20 nucleotides when keeping constant the sequence of the capture probe specific for the amplified target.

18. This is important in the context of a microarray because the hybridization conditions (temperature, buffer) for all of targets to be assessed on the array will be uniform while the optimal hybridization temperature for a given target could be different from another target. Enhancing the signal levels using spacers of the lengths recited in the claims enhances the ability to evaluate the binding of multiple targets under uniform conditions.

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19. The composition of the spacer is also very important and contributes to the unexpectedly high sensitivity observed. A spacer which is a nucleotide sequence gives a higher hybridization signal than a non-nucleotide spacer. The scientific explanation for such sensitivity improvement using as spacer a nucleotide sequence is not clear. One hypothesis is that the spacer plays a role not only in reducing the steric hindrance of the reaction near the surface but that the presence of non-specific DNA bases in the spacer plays an important role in increasing the extent of hybridization. This could result from non-specific interactions which are high enough to attract the target sequences to the capture probes but not so high as to result in non-specific binding under the experimental conditions are used. This hypothesis for the exceptionally good hybridization results is derived from the observation by the inventors that not all the DNA sequences are equally efficient as spacers.

20. The ability of nucleotide spacers at least 40 nucleotides in length to dramatically improve the extent of hybridization and the foregoing hypothesis were not known nor suggested at the time this application was filed.


21. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 14 July 2006

By: _____


Jean Remacle

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Eppendorf DualChip® GMO microarray for fast and safe analysis of genetically modified organisms in foodstuff

The new EU-wide regulations for gene technology identification define that more than 0.9% GMO (genetic modified organism) in foodstuff must be identified. Detection of these recognized GMOs is challenging in itself, and many new GMOs are currently in development or are waiting for their EU approval.

The DualChip GMO kit, based on Eppendorf's unique and innovative microarray technology, easily and conveniently enables the detection of GMOs in food in a highly standardized manner. As new GMOs are developed and approved for use, they can also be quickly and easily added to the existing microarray. For even quicker results, DualChip GMO can be used with the Eppendorf Silverquant® detection system to automatically analyze all GMO markers in parallel with high sensitivity and process safety.

Due to the microarray format, newly approved GMOs can easily be added. Eppendorf will initiate the technological validation by the EU within quarter 2 2006.

Article By

Eppendorf AG


Jens Behrens


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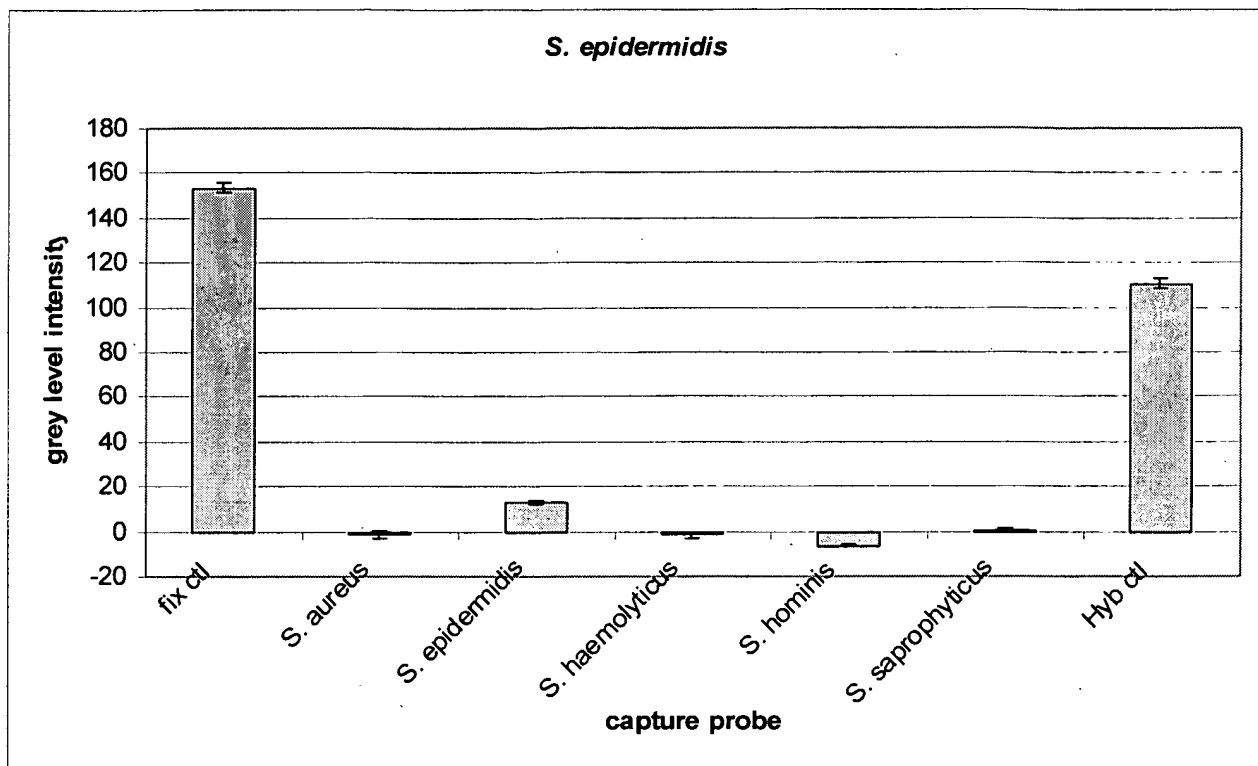


Exhibit 2: Detection of *S. epidermidis* after PCR and hybridization on DNA microarray having short specific capture probes for different *Staphylococcus* species according to the prior art (protocol of example 2 of the Specification). Fix.ctl is the fixation control for the probes. Hyb.ctl is the hybridization control.

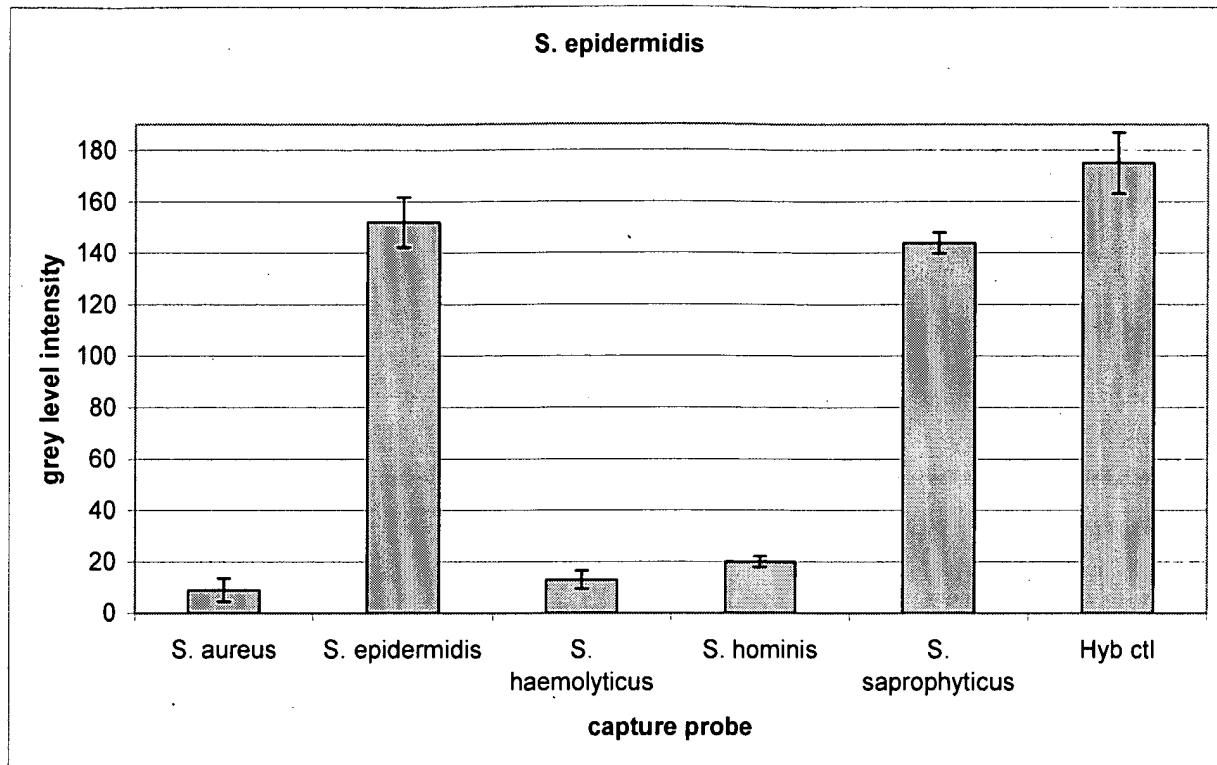


Exhibit 3: Detection of *S. epidermidis* after PCR and hybridization on DNA microarray having long specific capture probes (protocol of example 1 of the Specification). Hyb.ctl is the hybridization control.

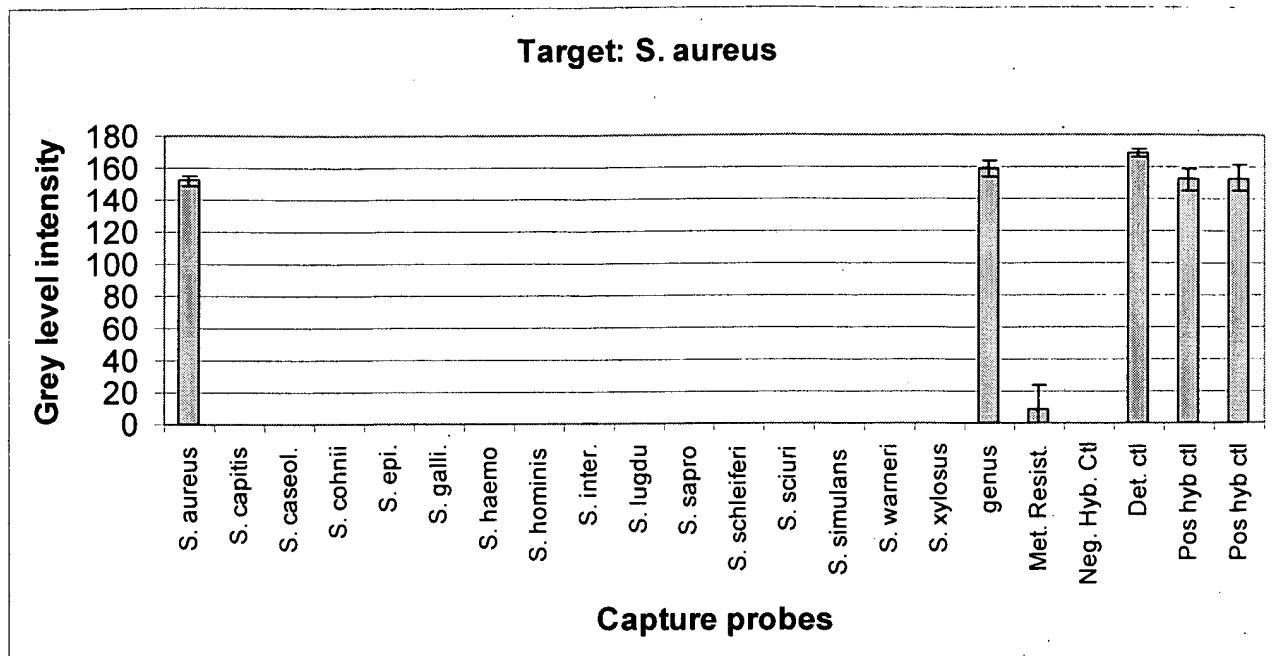


Exhibit 4: Detection of one bacteria species (*S. aureus*) after PCR using a consensus primer pair and hybridization on DNA microarray according to the invention on specific capture probes for different *Staphylococcus* species present or on a consensus probe for the *Staphylococcus* genus (protocol of examples 4 and 7 of the Specification). Det.ctl is the detection control. Poshyb ctl are the positive hybridization controls.

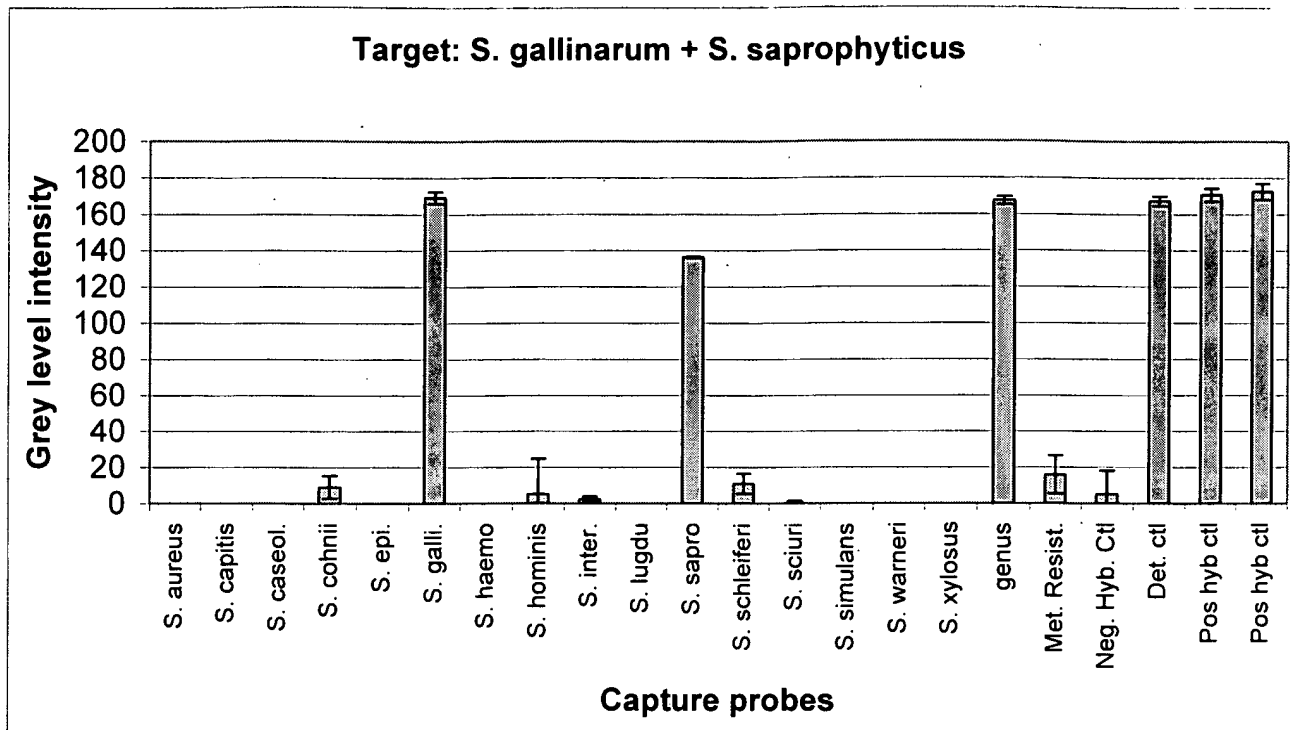


Exhibit 5: Simultaneous detection of two bacteria species (*S. gallinarum* and *S. saprophyticus*) after PCR using a unique consensus primer pair and on the microarray on specific capture probes for different *Staphylococcus* species present according to the invention or on a consensus probe for the *Staphylococcus* genus (protocol of examples 4 and 7 of the Specification). Det.ctl is the detection control. Poshyb.ctl are the positive hybridization controls

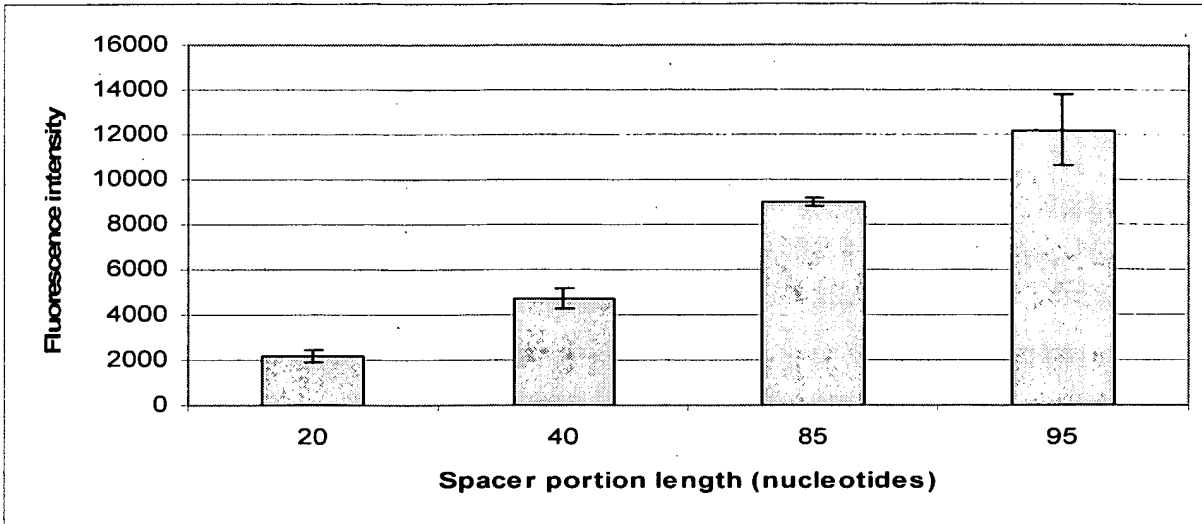


Exhibit 6: Effect of the spacer length on the hybridization yield (fluorescence intensity). The spacer length ranges from 20 to 95 bases with a constant sequence of 27 bases which is specific of the target molecule. The amplicons were obtained from amplification of *S. epidermidis FemA* gene by PCR

CV

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Education and profession:

1970	PhD Department of Science, University of Louvain, Belgium
1973	Postdoc, Rockefeller University, NY, USA
1974	Postdoc, UCSD, San Diego, USA
1974	Professor University of Namur, Belgium
1999	Founder of the spin off company "Advanced Array Technology"
2002	Director R-D Eppendorf Array Technologies (EAT)

Research Interests: Development of new DNA and protein biochips platform for genomic and for gene expression. Use of protein chips in complementation to the proteomic analysis by 2-D gel and mass spectra analysis. Applications for research as well as for diagnostic applications such as cancer prognostic and bacterial identification. Numerous collaborations with many research laboratories. Development and production of 8 biochips of gene expression analysis and genomic chips for the detection of bacteria.

Professional Experience:

Joined the University of Namur in 1974 and starting the laboratory for biochemistry and cell biology. Head of the laboratory since 1974.

Starting the research unit in cell biology (URBC) in 1997. The URBC is now composed of 65 researchers and technicians.

Member of many national and international committees, EEC expert in biotechnology programs. R-D Director of Eppendorf Array Technologies, S.A.

Publications

The author's scientific output consists of 351 research papers in peer-reviewed international journals and inventor of 47 filed patents

5 Publications 5 last years

1. I. Alexandre, S. Hamels, S. Dufour, J. Collet, N. Zammattéo, F. De Longueville, J.-L. Gala and J. Remacle, Colorimetric Silver Detection of DNA Microarrays, *Anal. Biochem.*, 295, 1-8, 2001 (Impact fact. 0.332).
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